Regulation of ligand binding to glycoprotein IIb–IIIa (integrin $\alpha_{IIb}\beta_3$) in isolated platelet membranes

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The major platelet integrin, glycoprotein IIb–IIIa, binds soluble fibrinogen only after platelet activation. To investigate the mechanism by which platelets convert glycoprotein IIb–IIIa into a functional fibrinogen receptor, we characterized the opening and closing of fibrinogen-binding sites in isolated platelet membranes and compared the regulatory properties of membrane-bound glycoprotein IIb–IIIa with those of the detergent-solubilized receptor. Basal fibrinogen binding to the membranes possessed many of the properties of fibrinogen binding to activated platelets; however, less than 10% of glycoprotein IIb–IIIa in the membranes was capable of binding fibrinogen. Preincubating the membranes with either an activating glycoprotein IIb–IIIa antibody or α-chymotrypsin increased fibrinogen binding. In contrast, agents that require intracellular mediators, such as platelet agonists, guanine-nucleotide-binding-protein activators and purified protein kinase C, did not stimulate fibrinogen binding to the membranes, suggesting that cytosolic factor(s) may be required for activation of the receptor in platelets. Occupancy of glycoprotein IIb–IIIa in the membranes with RGD (Arg-Gly-Asp)-containing peptides reversibly exposed neoantigenic epitopes and fibrinogen-binding sites in the receptor. These conformational changes required membrane fixation to be maintained following peptide removal. Similar results were obtained with purified glycoprotein IIb–IIIa incorporated into phospholipid vesicles, indicating that the resting state of the receptor is favoured in these environments. In contrast, when the conformation of detergent-solubilized glycoprotein IIb–IIIa was altered by exposure to RGD-containing peptides, the receptor remained active even after incorporation into phospholipid vesicles. These results demonstrate that platelet membranes are a useful model in which to study the regulation of glycoprotein IIb–IIIa and suggest that the environment surrounding the receptor may have a profound influence on this process.

INTRODUCTION

The membrane glycoproteins (GP) IIb and IIIa form a heterodimeric complex on the surface of platelets that binds the adhesive proteins fibrinogen, von Willebrand factor, vitronectin and fibronectin [1]. The interaction of GP IIb–IIIa with either fibrinogen or von Willebrand factor is required for platelet aggregation [1]. Fibrinogen binding is bivalent-cation-dependent and is mediated, at least in part, by RGD (Arg-Gly-Asp) sequences in the Aα chain of fibrinogen [2] and/or a region in the C-terminus of the γ-chain of fibrinogen [3,4,5]. GP IIb–IIIa belongs to a family of structurally related cell-adhesion receptors, the integrins, many of which recognize their ligands in an RGD-dependent manner [6,7]. In addition to recognizing large adhesive proteins, GP IIb–IIIa also binds small synthetic peptides containing either the RGD sequence [8] or the sequence HHLGGAKQAGDV (H12) derived from the C-terminus of the γ-chain of fibrinogen [3].

Fibrinogen binding to platelets is subject to tight metabolic control and occurs only after platelets are exposed to an activating agent, such as thrombin, ADP or thromboxane A_2 [9]. The rapid expression of fibrinogen-binding sites on platelets is the result of conversion of GP IIb–IIIa from a state that cannot bind fibrinogen into one that does. In the absence of fibrinogen, the binding-competent state of GP IIb–IIIa disappears, but can be restored upon exposure to a second agonist [10]. Activation of GP IIb–IIIa parallels other activation-dependent events in platelets, including alterations in phospholipid metabolism, mobilization of intracellular calcium, activation of protein kinases and rearrangement of the cytoskeleton. However, the precise series of intracellular events that are responsible for the conversion of GP IIb–IIIa into a functional fibrinogen receptor is not known.

GP IIb–IIIa exists in at least three distinct and interconvertible functional states: **the resting state** that cannot bind adhesive proteins, **the activated state** that can bind adhesive proteins and the **ligand-occupied state** that is expressed upon the binding of adhesive proteins. Although the physiological mechanism responsible for converting GP IIb–IIIa from a resting to an activated state within the platelet remains obscure, the activated state can be induced independently of intracellular mediators by the binding of certain GP IIb–/GP IIIa-specific monoclonal antibodies (mAb) [11,12,13] or RGD-containing peptides [14]. Moreover, many of the antibodies that recognize the ligand-occupied state also promote fibrinogen binding to the receptor, suggesting that the activated and ligand-occupied states are closely related [15]. The cytoplasmic domains of both GP IIb and GP IIIa have been implicated in the regulation of the affinity state of the receptor. A point mutation (Ser^{752}→Pro) in the cytoplasmic domain of GP IIIa blocks activation of the receptor in platelets [16], while deletion of the cytoplasmic domain of GP IIb renders the protein constitutively active when expressed recombinantly in cells [17].

While modulation of the fibrinogen binding capacity of both purified GP IIb–IIIa and GP IIb–IIIa in intact cells has been observed, relatively little is known about the regulation of the

Abbreviations used: GP, glycoprotein; GTP[S], guanosine 5′-[γ-thio]triphosphate; mAb, monoclonal antibody; PMSF, phenylmethanesulphonyl fluoride; PGI_2, prostacyclin; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; peptides, e.g. YRGDS, are given in the one-letter amino acid code; LIBS, ligand-induced binding sites.

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fibrinogen-binding properties of GP IIb–IIIa in purified platelet membranes. Phillips and Baughan [18] reported that the levels of fibrinogen binding to platelet membranes are much lower than those reported for fibrinogen binding to activated platelets. Thus platelet membranes may provide a model system in which to study potential mechanisms of regulation of GP IIb–IIIa. Platelet membranes have been of value in providing information on the regulation of other activation-dependent processes in platelets, including hormonal regulation of adenylate cyclase [19] and phospholipase C [20]. In the present study we characterized the properties of fibrinogen binding to isolated platelet membranes and investigated the mechanisms governing the opening and closing of binding sites on the fibrinogen receptor, GP IIb–IIIa.

EXPERIMENTAL

Materials

α-Chymotrypsin (bovine pancrease) and neuraminidase (from *Vibrio cholera*) were purchased from Sigma (St. Louis, MO, U.S.A.). Synthetic peptides were obtained from the following sources: GRGDSP and HHLGGAKQAGDV (H12) were from Biosearch (San Rafael, CA, U.S.A.), and YRGDS was from Pennisula Laboratories (Belmont, CA, U.S.A.) (peptides are given in the one-letter amino acid code). Saponin was purchased from Calbiochem (San Diego, CA, U.S.A) and streptolysin O was from the Burroughs Wellcome Co. (Greenville, NC, U.S.A.).

Antibodies

The GP IIIa-specific mAb AP3 [21] and the GP IIb-specific mAb AP4 were kindly supplied by Dr. Peter Newman and Dr. Thomas Kunicki of the Blood Center of Southeastern Wisconsin; the GP IIb–IIIa complex-specific mAb 10E5, which inhibits platelet aggregation and fibrinogen binding [22], was generously supplied by Dr. Barry Coller, State University of New York, Stony Brook, NY, U.S.A.; the GP IIIa-specific mAb 62, which induces the exposure of fibrinogen-binding sites on GP IIb–IIIa [13], the GP IIIa-specific mAb LIBS1, which preferentially recognizes the ligand-occupied state of GP IIb–IIIa [15], and the GP IIb-specific mAb PMI-1 [23], which recognizes an epitope at the C-terminus of the heavy chain of GP IIb, were provided by Dr. Mark Ginsberg, the Scripps Research Institute, La Jolla, CA, U.S.A.; the activation-dependent anti-(GP IIb–IIIa) mAb PAC-1 [24] was generously given by Dr. S. Shattil; rabbit polyclonal antibodies to GP IIb–IIIa were obtained from Dr. David Phillips, COR Therapeutics.

Fibrinogen purification

Fibrinogen was purified from fresh-frozen plasma by the glycine precipitation method of Kazal et al. [25] with an additional gelatin–Sepharose chromatography step to remove contaminating fibronectin. The concentration of fibrinogen was determined spectrophotometrically (A_{280}), assuming an absorption coefficient ($A^{1\%}_{280}$) of 15.1 and a molecular mass of 340 kDa. Fibrinogen was centrifuged (11600 *g*) for 5 min before use.

Iodination of YRGDS and proteins

Radioiodinations were catalysed by chloramine-T (0.05 mg/ml) and stopped with sodium metabisulphite (0.05 mg/ml). Unincorporated $Na^{125}I$ was separated from radiolabelled YRGDS by gel filtration on Sephadex G-10 (Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.) and from radioiodinated proteins by gel filtration on PD-10 columns (Pharmacia). The specific activity was $\sim 5 \times 10^{16}$ and $\sim 1 \times 10^{18}$ c.p.m./mol for ^{125}I-labelled YRGDS and ^{125}I-labelled proteins respectively.

Platelet isolation and functional analysis

Platelet-rich plasma from acid-citrate/dextrose-anticoagulated human blood was centrifuged at 800 *g* for 20 min, and the resulting platelet pellet was resuspended in Tyrode's solution (5.5 mM glucose/138 mM NaCl/12 mM $NaHCO_3$/0.36 mM NaH_2PO_4, and 10 mM Hepes, pH 7.4) containing 0.35% BSA and 150 nM prostacyclin (PGI_2). Platelets were washed twice by centrifugation at 800 *g* for 15 min. Between spins, the platelets were incubated at 37 °C for a minimum of 15 min. Platelets were resuspended at 3×10^8/ml in Tyrode's solution containing 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.35% BSA and tested for their ability to aggregate to one of the following: 0.1 unit/ml thrombin; 10 μM phorbol 12-myristate 13-acetate (PMA); or 0.6 μM U46619 (a thromboxane A_2 mimetic).

Fibrinogen-binding assays were performed in a total volume of 200 μl containing Tyrode's solution with 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.35% BSA. Binding was initiated by the addition of a platelet agonist and ^{125}I-fibrinogen. Bound fibrinogen was separated from free fibrinogen by centrifuging (13500 *g* for 2 min) 50 μl of the reaction mixture through 400 μl of 20% (w/v) sucrose, aspirating the liquid, and quantifying the amount of radioactivity associated with the platelet pellet. In some instances suspensions of washed platelets were incubated with chymotrypsin for 20 min at room temperature or mAb 62 for 30 min at 37 °C before assessing the ability of the platelets to aggregate or to bind fibrinogen. Chymotrypsin was inactivated by the addition of 200-fold molar excess of phenylmethanesulphonyl fluoride (PMSF) to enzyme.

Platelet membrane preparation

Outdated platelets (American Red Cross) were isolated from platelet-rich plasma by centrifugation (700 *g*, 20 min) and washed twice in 120 mM NaCl/13 mM trisodium citrate/30 mM dextrose, pH 7.0. Washed platelets were resuspended in buffer A (150 mM NaCl/10 mM Tris, pH 7.4) and placed in the pressure chamber of a Parr cell disruption bomb (Parr Instrument Co., Moline, IL, U.S.A.). The chamber was filled with nitrogen to a pressure of 8280 kPa (1200 lbf/in^2) and maintained at 22 °C for 30 min. The contents were discharged into the same buffer with final concentrations of 0.1 mM PMSF, 10 μg/ml aprotonin and 0.1 mg/ml leupeptin. The resultant suspension was centrifuged at 100000 *g* for 30 min at 4 °C. The pellet was resuspended in buffer A, layered over 27% sucrose in buffer A and centrifuged at 27000 rev./min for 3 h in an SW 28 Beckman rotor. The membranes were retrieved from the interface of the sucrose solution, resuspended in buffer A with 1 mM $CaCl_2$, and centrifuged for 1 h at 100000 *g* at 4 °C.

Enzyme assays

For sialic acid analysis, platelet membranes were treated with 0.1 unit/ml neuraminidase for 120 min at 37 °C with or without prior solubilization in 0.05% Triton X-100. Released sialic acid was measured by the thiobarbituric acid assay described by Warren [26].

Phosphodiesterase activity was measured by hydrolysis of 0.1 mM bis-(*p*-nitrophenyl) phosphate in 30 mM $MgCl_2$/30 mM

Tris, pH 8.8. The reaction was initiated by the addition of membranes that had been preincubated for 1 min with or without 0.1% Triton X-100. The A_{420} of the solution was read after the addition of membranes and again after a 30 min incubation at 37 °C. The difference between the two readings is a measure of phosphodiesterase activity in the membranes [27].

Ligand binding to purified platelet membranes

Platelet membranes were incubated in a total volume of 200 μl containing 100 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5% BSA, 50 mM Tris, pH 7.4, and ^{125}I-labelled ligand for 1 h at room temperature. The reactions were terminated by filtering 50 μl aliquots through polycarbonate filters (0.2 μm pore size; Nuclepore, Cambridge, MA, U.S.A.). The filters were washed three times with 1 ml of ice-cold buffer (100 mM NaCl/1 mM $CaCl_2$/0.02% NaN_3/50 mM Tris, pH 7.4), and the radioactivity associated with the filters was quantified. Non-specific binding was defined as the amount of ^{125}I-ligand bound in the presence of 3 μM non-radioactive ligand. In some instances, equilibrium dialysis was also performed as previously described [45] to measure the binding of ^{125}I-peptide to the membranes.

Chymotrypsin treatment of platelet membranes

Platelet membranes were incubated in 50 mM Tris (pH 7.4)/100 mM NaCl/1 mM $CaCl_2$ with various concentrations of chymotrypsin for 30–60 min at room temperature. The reactions were terminated by the addition of 200-fold molar excess of PMSF. Aliquots of the chymotrypsin-treated membranes were incubated with ^{125}I-fibrinogen in the binding assay or solubilized in SDS sample buffer for gel electrophoresis.

Protein kinase C (PKC) purification and membrane protein phosphorylation

Rat brain PKC was purified by sequential DEAE-cellulose, threonine–Sepharose and phenyl-Sepharose chromatography as described by Kitano et al. [28] PKC activity was assayed in 20 mM Tris (pH 7.4)/5 mM magnesium acetate/10 μM [γ-^{32}P]ATP/0.1 mM $CaCl_2$/40 μg/ml phosphatidylserine/2 μg/ml diolein (dioleoylglycerol). The specific activity of the purified enzyme was 0.7 μmol of phosphate transferred/min per mg of protein using histone H1 (200 μg/ml) as the substrate.

Membrane phosphorylation reactions were conducted in 10 mM Tris (pH 7.4)/5 mM $MgCl_2$/1 mM $CaCl_2$/100 μM ATP for 60 min at 37 °C with or without the addition of purified 10 μl PKC (0.1 mg/ml). In some cases the membrane-permeabilizing agents saponin (1–100 μg/ml) or streptolysin O (1 unit/ml) were included in the binding reaction. Previous studies indicated that 30 μg/ml saponin or 1 unit/ml streptolysin O were sufficient to permeabilize intact platelets, as determined by measuring platelet aggregation in response to the membrane-impermeable reagent guanosine 5′-[γ-thio]triphosphate (GTP[S]). The phosphorylation of platelet membrane proteins was monitored by including [γ-^{32}P]ATP in the reaction, solubilizing the proteins in SDS sample buffer and subjecting them to SDS/PAGE. ^{32}P-labelled bands were revealed by autoradiography.

GRGDSP incubations and membrane fixations

Membranes were incubated without or with GRGDSP for 15 min at room temperature, diluted in Buffer A with 1 mM $CaCl_2$, centrifuged at 13000 *g* for 60 min and initially resuspended in the same buffer. Ligand-binding studies were performed with buffers as described for ligand binding to platelet membranes. For fixation studies, membranes incubated with or without GRGDSP were diluted with an equal volume of 1% paraformaldehyde in 0.075 M sodium phosphate (pH 7.4)/0.075 M NaCl, incubated for an additional 60 min at room temperature and neutralized with an equal volume of 500 mM NH_4Cl in 10 mM sodium phosphate/150 mM NaCl. The fixed membranes were washed, resuspended as described above, and ligand-binding studies were performed as described above.

GP IIb–IIIa purification and incorporation into phospholipid vesicles

GP IIb–IIIa was purified from outdated platelets that were lysed in 50 mM octyl glucoside/10 mM Hepes (pH 7.4)/150 mM NaCl/2 mM $CaCl_2$/1 mM $MgCl_2$. After centrifugation at 30000 *g* for 15 min, the platelet lysate was applied to a concanavalin A affinity column and the bound glycoproteins were eluted with 500 mM α-methylmannose in the above buffer [29]. The fractions containing the peak of GP IIb–IIIa were pooled, applied to sequential heparin–Sepharose and gelatin–Sepharose columns and concentrated approx. 5-fold to 1 mg/ml by ultrafiltration. GP IIb–IIIa, judged to be > 85% pure by scanning densitometry of SDS/polyacrylamide gels, was incorporated into 70% phosphatidylserine/30% phosphatidylcholine phospholipid vesicles [1:2.9 (w/w) protein/lipid ratio] by detergent dialysis as described in [30]. Ligand binding to the vesicles was performed as described for ligand binding to platelet membranes.

SDS/PAGE and Western blotting

Proteins were solubilized in 2% SDS/20% glycerol/0.125 M Tris, pH 6.8, in the presence (reducing conditions) or absence (non-reducing conditions) of 2% (v/v) β-mercaptoethanol and separated on SDS/7.5% or 5–20% polyacrylamide gels [31]. The gels were stained with Coomassie Brilliant Blue R250 or equilibrated in transfer buffer [50 mM Tris/(pH 9.2)/55 mM glycine/1 mM SDS/20% methanol] for Western blotting. Proteins were transferred to nitrocellulose with a Gelman semi-dry graphite-electrode apparatus. The nitrocellulose was incubated with primary antibody in 100 mM Tris/0.9% NaCl/0.1% Tween 20 for 30 min at room temperature, and the bound Abs were detected with the Vectastain ABC horseradish peroxidase detection system (Vector Laboratories, Burlingame, CA, U.S.A.).

RESULTS AND DISCUSSION

Properties of purified platelet membranes

Isolated platelet membranes were prepared by nitrogen cavitation of washed platelets followed by sucrose-density sedimentation. Electron micrographs indicate that this procedure yields relatively pure intact platelet membranes (results not shown). The accessibility of the outer surface of the membranes was assessed by treating the membranes with neuraminidase in the presence or absence of 0.05% Triton X-100. Neuramidase cleaves sialic acid residues, which are located exclusively on the extracellular portions of membrane proteins. In intact and solubilized membranes, neuraminidase hydrolysed similar amounts of sialic acid (53.4 and 53.9 nmol/mg of membrane protein respectively), demonstrating that the extracellular face of the membranes is exposed. The accessibility of the inner surface of the membranes was evaluated by measuring phosphodiesterase activity in the presence and absence of 0.1% Triton X-100. Phosphodiesterase

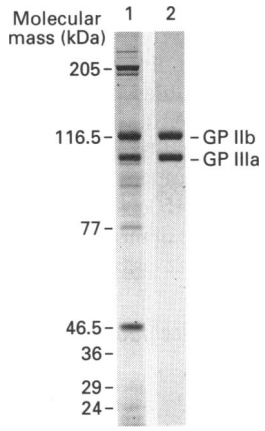

Figure 1 Protein content of purified platelet membranes

Platelet membranes (25 μg; lane 1) and purified GP IIb-IIIa (2.5 μg; lane 2) were solubilized in 2% SDS, reduced with 2% β-mercaptoethanol and electrophoresed through a 5–20%-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue; the positions of the molecular-mass markers are indicated.

activity was similar with or without membrane solubilization (0.62 units/mg of protein versus 0.78 unit/mg of protein respectively), indicating that the inner surface of the membranes is accessible to small molecules such as bis-(*p*-nitrophenyl) phosphate.

The protein content of the membranes is shown in the Coomassie Blue-stained SDS/polyacrylamide gel in Figure 1. Two heavily stained proteins in the membranes co-migrate with purified GP IIb and GP IIIa and react with anti-(GP IIb–IIIa) antibodies on Western blots. To estimate the amount of complexed GP IIb–IIIa in these membranes, binding of the GP IIb–IIIa complex-specific mAb 10E5 was quantified; 5.84×10^{-10} mol of ^{125}I-10E5 were bound per mg of membrane protein, which represents approx. 15% of total membrane protein.

Fibrinogen-binding properties of platelet membranes

The amount of ^{125}I-fibrinogen binding to the membranes increased linearly as increasing concentrations of membrane protein (5–50 μg) were included in the assay. Fibrinogen binding (10 nM) to the membranes was time-dependent and reached steady state within approx. 30 min at room temperature (results not shown). Excess non-radioactive fibrinogen competed for radiolabelled fibrinogen binding, indicating that fibrinogen binding to the membranes is saturable. Maximal inhibition was observed when a 100-fold excess of non-radioactive fibrinogen was included in the binding reaction (IC_{50} ~ 100 nM; Figure 2a).

Competition binding studies with three different agents that interact with GP IIb–IIIa suggest that fibrinogen is binding to GP IIb–IIIa in the membranes. First, the GP IIb–IIIa-specific mAb 10E5, which blocks fibrinogen binding to whole platelets [22], inhibited fibrinogen binding to the membranes (Figure 2b). Moreover, the synthetic peptides GRGDSP (Figure 2c) and H12 (Figure 2d) competed for fibrinogen binding to the membranes, while the control peptide GRGESP had no effect. The concentrations of GRGDSP and H12 required for 50% inhibition of ^{125}I-fibrinogen binding were 50 and 20 μM respectively. These peptides display similar potencies for inhibition of

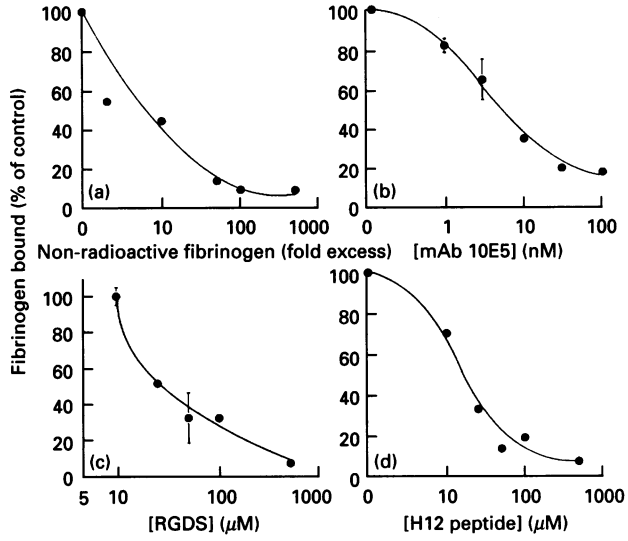

Figure 2 Inhibition of ^{125}I-fibrinogen binding to platelet membranes

Platelet membranes (25 μg) were incubated for 60 min with ^{125}I-fibrinogen in the presence of various concentrations of non-radioactive fibrinogen (**a**), the GP IIb-IIIa specific mAb 10E5 (**b**), the synthetic peptide GRGDSP (**c**) or the synthetic H12 peptide (**d**) corresponding to the C-terminus of the γ-chain of fibrinogen. Data are expressed as percentages of maximal ^{125}I-fibrinogen bound.

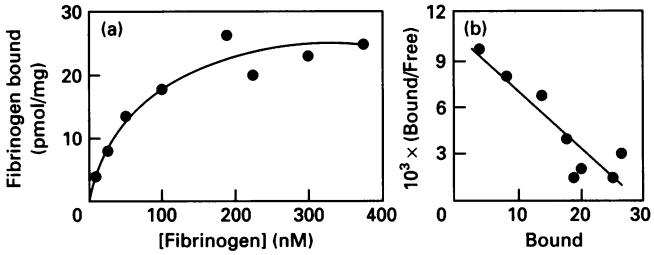

Figure 3 Saturation isotherm and Scatchard analysis of ^{125}I-fibrinogen binding to platelet membranes

(**a**) Platelet membranes (25 μg) were incubated for 60 min at room temperature with increasing concentrations of ^{125}I-fibrinogen (10–400 nM). Non-specific binding was defined with 3 μM non-radioactive fibrinogen, with the amount of saturable binding ranging from 60 to 80% of total binding. (**b**) Analysis of the same data by the method of Scatchard [53]. Data are plotted as pmol of radioligand bound/mg (abscissa) versus bound ligand divided by free-ligand concentration (ordinate). The data are representative of results obtained for ten different membrane preparations in which the affinity constant (K_d) for fibrinogen binding ranged from 15 to 50 nM and the maximal binding capacity (B_{max}) for fibrinogen was less than 60 pmol/mg of protein.

^{125}I-fibrinogen binding to agonist-stimulated platelets, with reported IC_{50} values of 30–50 μM for RGD peptides [32,33] and 15–75 μM for H12 [3,34,35]. Thus fibrinogen binding to the membranes displays many of the properties of fibrinogen binding to whole platelets and to purified GP IIb–IIIa.

Equilibrium binding studies demonstrated that ^{125}I-fibrinogen binding is saturable. Scatchard transformation of the data revealed that fibrinogen binds with high affinity, apparently to a single class of sites (Figure 3). In ten different membrane preparations the dissociation constant (K_d) for fibrinogen binding ranged from 15 to 50 nM, compared with reported values of 50–500 nM for fibrinogen binding to whole platelets [1] and 15 nM for fibrinogen binding to platelet membranes [18]. The

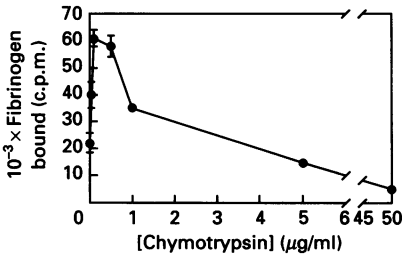

Figure 4 Chymotrypsin-induced fibrinogen binding to membranes

Membranes were preincubated with various concentrations of chymotrypsin (0–50 μg/ml) for 60 min at room temperature and the reaction was terminated by the addition of 1 mM PMSF. ^{125}I-fibrinogen binding (30 nM) to the membranes was assayed as described in the Experimental section. Results are means ± S.E.M. for triplicate samples.

maximal binding capacity ($B_{max.}$) of the membranes was < 60 pmol of fibrinogen bound/mg of protein. A similar number of binding sites were observed in membranes prepared from platelets disrupted by sonication. Thus the density of fibrinogen binding sites is only a fraction of the total GP IIb–IIIa (~ 600 pmol/mg of protein) in the membranes, indicating that only a small proportion (< 10%) of the receptors are functional. In this manner the fibrinogen-binding properties of the membranes are similar to those of resting platelets in that substantial amounts of GP IIb–IIIa are present but unable to bind fibrinogen.

Exposure of fibrinogen-binding sites in membranes by the serine proteinase chymotrypsin

The low levels of fibrinogen binding to the membranes could be explained if the membrane isolation procedure rendered GP IIb–IIIa non-functional. We therefore sought to determine whether the fibrinogen-binding capacity of GP IIb–IIIa in the membranes could be increased in a manner independent of intracellular mediators. Chymotrypsin treatment of intact platelets in the absence of intracellular metabolic events exposes cryptic fibrinogen-binding sites on GP IIb–IIIa by either proteolysis of GP IIb–IIIa or an accessory molecule [36,37]. We observed spontaneous aggregation of platelets in the presence of exogenous fibrinogen after exposure of platelets to 30–150 μg/ml chymotrypsin for 20 min at room temperature, which correlated with an increase in ^{125}I-fibrinogen binding to chymotrypsin-treated platelets (results not shown).

We next examined the ability of chymotrypsin to increase the fibrinogen binding properties of membranes. When membranes were incubated with increasing concentrations of chymotrypsin and then assayed for fibrinogen binding, a 3-fold increase in the amount of ^{125}I-fibrinogen bound was observed in membranes pretreated with 0.05–0.5 μg/ml chymotrypsin (Figure 4). Pretreatment with higher concentrations of chymotrypsin (1–100 μg/ml) dose-dependently decreased fibrinogen binding (Figure 4).

Western-blot analysis of chymotrypsin-treated membranes indicated that the chymotrypsin-induced increase in fibrinogen binding did not correlate with digestion of either GP IIb or GP IIIa. The mobility of non-reduced (Figure 5a, lanes 1–5) and reduced (Figure 5b, lanes 1–4) GP IIb was unaltered following the exposure of membranes to 0.05–1 μg/ml chymotrypsin, conditions under which an increase in fibrinogen binding was observed (Figure 4). Immunoblot analysis with the GP IIb-specific mAbs AP4 and PMI-1 (Figure 5c, lanes 4–9) revealed that GP IIb was converted into a lower-molecular-mass form (~ 80 kDa) in membranes exposed to 10 μg/ml chymotrypsin. At concentrations of chymotrypsin required for exposure of fibrinogen-binding sites in the membranes, we did not observe the limited proteolysis of GP IIb that results in the release of the PMI-1 epitope and reportedly coincides with chymotrypsin-induced fibrinogen binding to intact platelets [38], even when we separated the proteins on 5%-polyacrylamide gels (results not shown). Differences in assay systems may account for our inability to detect this reaction.

As in intact platelets, GP IIIa in membranes was more sensitive to proteolysis by chymotrypsin. Limited cleavage of the proteolytically sensitive disulphide loop in GP IIIa to generate a 110 kDa proteolytic product was observed at 0.1 μg/ml chymotrypsin (Figure 5a, lanes 3) and was more prominent at higher doses (1–10 μg/ml) of chymotrypsin (Figure 5a, lanes 5–7; Figure 5c, lanes 1–3). High doses of chymotrypsin (> 10 μg/ml) produced 68 and 51 kDa forms of GP IIIa (Figure 5a, lane 8; Figure 5b, lanes 6–8; Figure 5c, lane 3). The identity of the

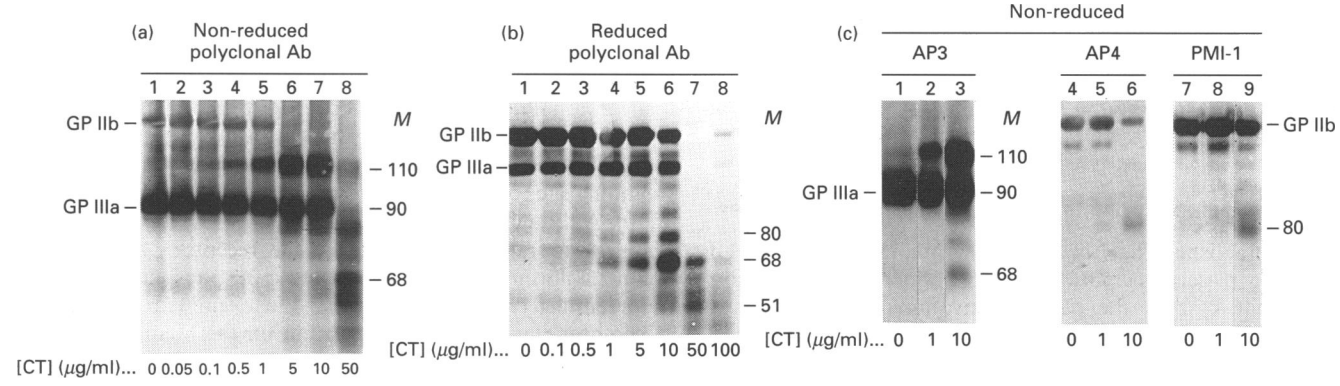

Figure 5 Western-blot analysis of GP IIb–IIIa in chymotrypsin-treated membranes

Membranes (25 μg) were incubated with the indicated concentration of chymotrypsin (0.05–100 μg/ml) for 60 min at room temperature, at which time the reaction was terminated by the addition of PMSF. The proteins were separated on a SDS/7.5%-polyacrylamide gel run under non-reducing (**a** and **c**) or reducing conditions (**b**). GP IIb and GP IIIa were revealed by Western blotting with anti-(GP IIb–IIIa) polyclonal Abs (**a** and **b**), with the GP IIIa-specific mAb AP3 (**c**, lanes 1–3), with the GP IIb-specific mAb AP4 (**c**, lanes 4–6) or with the GP IIb-specific mAb PMI-1 (**c**, lanes 7–9). *M*, molecular masses in kDa.

110 kDa and the 68 kDa proteins was confirmed by immunoreactivity with the GP IIIa-specific mAb AP3 (Figure 5c, lanes 1–3). Although initial reports correlated the acquisition of fibrinogen-binding properties of chymotrypsin-treated platelets with the generation of a 68 kDa fragment of GP IIIa [39], our results indicate that the appearance of this fragment occurs at higher concentrations of chymotrypsin than are needed to stimulate fibrinogen binding. Indeed, as reported by others investigating chymotrypsin-treated platelets [40], we find that the presence of the 68 kDa fragment correlates with a decrease in fibrinogen binding to the membranes. The 68 kDa fragment is generated by proteolysis of a large disulphide-bonded loop in GP IIIa that contains the putative RGD-binding site [41]. Loss of this region of GP IIIa may account for the decline in fibrinogen binding to membranes.

Our inability to correlate digestion of either GP IIb or GP IIIa with the exposure of additional fibrinogen-binding sites in membranes could arise if our Western-blot analysis was not sensitive enough to detect either minor alterations in the structure of the proteins or alterations in a subpopulation of the receptors. Alternatively, chymotrypsin may expose fibrinogen-binding sites in membranes by cleaving a molecule associated with GP IIb–IIIa. This hypothesis is supported by the finding that, following chymotrypsin digestion of intact platelets, the proteolytic products of GP IIb–IIIa are unable to bind either immobilized fibrinogen [42] or RGD peptides [41].

Induction of fibrinogen binding to membranes by a GP IIIa-specific mAb

Several mAbs directed against GP IIb and/or GP IIIa induce fibrinogen binding to GP IIb–IIIa, apparently by shifting the receptor from a low- to a high-affinity state [11–13]. One such mAb, 62 [13], was used as a second tool to stimulate fibrinogen binding to membranes. Incubation of intact platelets with mAb 62 (3–6 μM) resulted in aggregation upon addition of fibrinogen, without the need for a platelet agonist (results not shown). When preincubated with membranes, mAb 62 dose-dependently increased fibrinogen binding to GP IIb–IIIa (Figure 6). At the highest concentration tested (1.8 μM), mAb 62 promoted a 5-fold increase in fibrinogen-binding levels. The activating mAb 62 also increased the number of binding sites for PAC-1, a GP IIb–IIIa-specific mAb that recognizes an epitope exposed only in the activated form of the receptor. Maximally effective concentrations of mAb 62 (10 μM) stoichiometrically exposed PAC-1-binding sites in GP IIb–IIIa in the membranes ($B_{max.}$ > 650 pmol/mg of protein; results not shown). Thus mAb 62 induces a ligand-binding competent form of GP IIb–IIIa in membranes, presumably by directly affecting the conformation of the receptor.

Effects of platelet activators on fibrinogen binding to membranes

Having established that the fibrinogen-binding activity of GP IIb–IIIa in platelet membranes could be increased under conditions that by-pass the platelet-activation process, we next examined the effects of platelet activators on the fibrinogen-binding capacity of membranes. Fibrinogen binding to platelets is stimulated by the platelet agonists thrombin and U46619 (a thromboxane A_2 analogue), presumably by receptor-mediated activation of a guanine-nucleotide-sensitive binding protein (G-protein) and a subsequent cascade of intracellular events involving PKC. In contrast with their effects on intact platelets, the platelet agonists ADP, thrombin and U46619 did not stimulate fibrinogen binding to the membranes, either when added alone

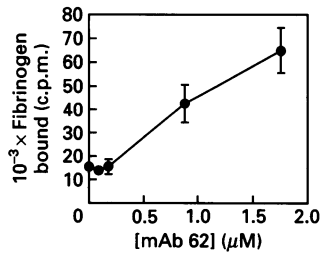

Figure 6 Induction of fibrinogen binding by a GP IIb–IIIa-activating mAb

Binding of ^{125}I-fibrinogen (30 nM) to platelet membranes was measured in the absence or presence of various concentrations of activating mAb 62. Binding reactions were allowed to proceed for 60 min at room temperature as described in the Experimental section. Results are means ± S.E.M. for triplicate samples.

Table 1 Effects of platelet activators on fibrinogen binding to membranes

Platelet membranes were incubated with the indicated platelet activators and ^{125}I-fibrinogen for 1 h at room temperature. The amount of fibrinogen bound was measured as described in the Experimental section. The combined results from two separate experiments performed in triplicate are reported.

Agent	Fibrinogen bound (pmol/mg of membrane protein)
Control	54.0 ± 2.0
U46619 (0.6 μM)	55.4 ± 5.4
ADP (10 μM)	26.7 ± 5.0
Thrombin (0.1 unit/ml)	30.5 ± 5.1
GTP[S] (50 μM)	43.0 ± 3.5
U46619 (0.6 μM) + GTP[S] (50 μM)	49.5 ± 8.8
PMA (5 μM)	45.8 ± 8.8
ATP (100 μM)	50.3 ± 15
ATP (100 μM) + PKC (1 μg)	24.6 ± 5.7
mAb 62 (6 μM)	435 ± 11

or in combination with the G-protein activators NaF, GTP or GTP [S] (Table 1). Enzymic analysis revealed that both the inner and outer surfaces of the membranes were accessible to small molecules (see above), eliminating one explanation for these results. In platelet membrane preparations similar to ours, receptor- and G-protein-mediated regulation of adenyl cyclase has been observed [19], indicating that at least part of the stimulus-coupling machinery is intact in this system. Our findings suggest that agonist-receptor occupancy cannot directly regulate GP IIb–IIIa activity in the membranes.

In agonist-stimulated platelets, the exposure of fibrinogen-binding sites on GP IIb–IIIa correlates closely with PKC activity [10]. Although direct phosphorylation of GP IIb–IIIa does not appear to regulate the state of the receptor [43], PKC may control fibrinogen binding by altering the phosphorylation of an unidentified regulatory protein. We examined the effects of membrane protein phosphorylation on the binding capacity of GP IIb–IIIa. Platelet membranes possess endogenous protein kinase activity, as detected by the incorporation of [^{32}P]phosphate into specific membrane proteins (Figure 7, lane 1). The addition of PKC to membranes resulted in [^{32}P]phosphate incorporation into at least one additional protein with a molecular mass of approx. 70 kDa (Figure 7, lane 2). Under conditions where specific protein phosphorylation was observed, fibrinogen binding to the membranes was not promoted by treatment of the

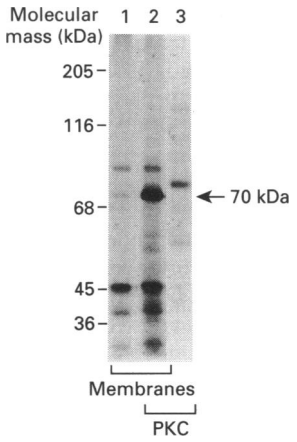

Figure 7 Platelet membrane phosphorylation

Membranes were incubated for 30 min at 37 °C with 10 μM [γ-^{32}P]ATP (lane 1) or 10 μM [γ-^{32}P]ATP and purified PKC (lane 2). Lane 3, purified PKC was incubated with 10 μM [γ-^{32}P]ATP in the absence of platelet membranes. The arrow points to a 70 kDa protein specifically phosphorylated by PKC. The positions of molecular-mass markers are indicated.

membranes with phorbol esters, ATP or purified PKC (Table 1). To rule out the possibility that substrates in the membranes were inaccessible to PKC, the experiments were repeated in the presence of the membrane-permeabilizing agents saponin and streptolysin O. The permeabilizing agents did not alter the phosphorylation patterns or basal fibrinogen binding to the membranes; moreover, they did not result in higher fibrinogen binding with the addition of PKC (results not shown). Taken together, these results suggest that the affinity state of GP IIb–IIIa may be controlled by phosphorylation/dephosphorylation of (a) cytoplasmic component(s) that is (are) lost during the membrane isolation procedure.

YRGDS binding to membranes

RGD-containing peptides reportedly interact with resting platelets [44] and purified GP IIb–IIIa [45]. The ability of GRGDSP to compete for fibrinogen binding to membranes (Figure 2c) suggested that RGD-containing peptides could potentially interact with GP IIb–IIIa in the membranes. Thus the binding of ^{125}I-YRGDS to the membranes was characterized. The binding of ^{125}I-YRGDS to the membranes increased over time and approached steady state within 1 h at room temperature and within 6 h at 4 °C. The binding was specific and saturable in that excess non-radioactive YRGDS, GRGDSP or bivalent-cation chelation inhibited ^{125}I-YRDGS binding (results not shown). Determination of the equilibrium binding constants for ^{125}I-YRGDS binding to the membranes revealed that the affinity constant (K_d) is approx. 150 μM (Figure 8). Thus the affinity of membrane for YRGDS, as determined by equilibrium-binding analysis, is in the range of the affinity for GRDGSP, as determined by inhibition of fibrinogen binding (IC_{50} approx. 50 μM). The maximal binding capacity ($B_{max.}$) of membranes for YRGDS was 700 pmol/mg of protein, which corresponds to the total GP IIb–IIIa content of the membranes (600 pmol/mg of protein). Thus, as in resting platelets, GP IIb–IIIa in the membranes appears to be capable of interacting stoichiometrically with short peptide sequences but not with intact fibrinogen. When incubated with low concentrations of ^{125}I-YNRGDS (20 μM), platelet membranes bound a larger quantity of peptide in the presence of activating mAb 62 (results not shown), suggesting that changes in the state of GP IIb–IIIa may affect the receptor's affinity for peptides.

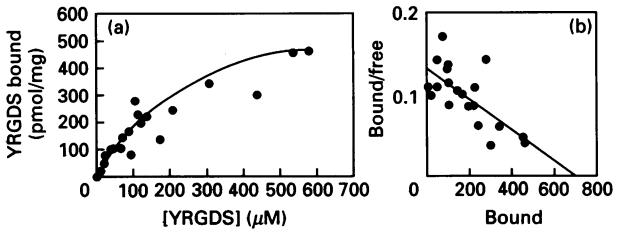

Figure 8 Analysis of ^{125}I-YRGDS binding to membranes

Membranes (25 μg) were incubated with ^{125}I-YRGDS and various concentrations of nonradioactive YRGDS for 60 min at room temperature. (**a**) The data were plotted as total YRGDS added (abscissa) versus bound YRGDS (ordinate). (**b**) Data were replotted according to the method of Scatchard [53] and are plotted as pmol of YRGDS bound/mg (abscissa) versus bound ligand divided by free ligand (ordinate). The data are the combined results from three separate experiments. The affinity constant (K_d) for YRGDS binding to membranes was 200 μM, and the maximal binding capacity ($B_{max.}$) was 700 pmol/mg of protein.

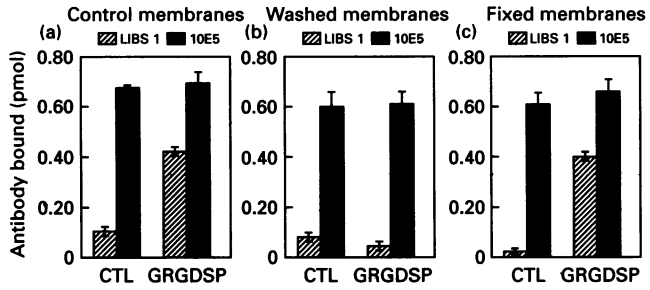

Figure 9 Exposure of mAb LIBS 1-binding sites in membranes by GRGDSP

The ability of GRGDSP to expose LIBS in GP IIb–IIIa in membranes was assessed by measuring the binding of ^{125}I-mAb LIBS 1 (80 nM; ▨) to membranes that were pretreated with buffer ('CTL') or 1 mM GRGDSP for 15 min at room temperature (**a**), to membranes that were pretreated with buffer or GRGDSP as above and washed to remove the peptide as described in the Experimental section (**b**), and to membranes that were pretreated with buffer or peptide, fixed in 0.5% paraformaldehyde for 1 h and then washed to remove peptide (**c**). ^{125}I-mAb 10E5 binding (200 nM; ■) was performed under each of the three conditions to measure the total amount of exposed GP IIb–IIIa.

Effects of RGD peptide occupancy on the conformational state of GP IIb–IIIa

Previous reports indicate that occupancy of GP IIb–IIIa with RGD-containing peptides alters the conformation of the receptor [46] and exposes neoantigenic sites termed 'ligand-induced binding sites' (LIBS) [47,48]. In membranes, RGD-containing peptides similarly altered the conformation of GP IIb–IIIa to expose LIBS that were recognized by the conformationally sensitive anti-(GP IIIa) mAb LIBS 1 (Figure 9a). The conformational changes in GP IIb–IIIa induced by the peptide were reversed upon removal of the peptide (Figure 9b), but could be maintained if the membranes were incubated with GRGDSP and subsequently fixed with paraformaldehyde (Figure 9c).

Du and co-workers [14] demonstrated that the conformation of ligand-occupied GP IIb–IIIa appears to be similar to the conformation of activated GP IIb–IIIa, in that pre-exposure of

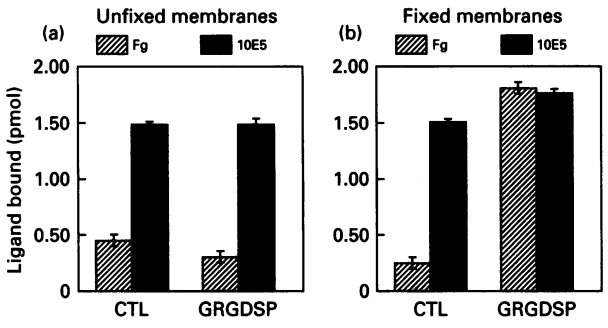

Figure 10 ^{125}I-fibrinogen (Fg) and ^{125}I-mAb 10E5 (10E5) binding to membranes pretreated with GRGDSP

(**a**) Membranes (35 μg) were pretreated without ('CTL') or with the peptide GRGDSP (1 mM) for 15 min at room temperature. The reaction was terminated by dilution and centrifugation as described in the Experimental section. Binding of ^{125}I-fibrinogen (200 nM; ▨) or ^{125}I-mAb 10E5 (200 nM; ■) to the resuspended membranes was quantified. (**b**) Membranes were incubated without or with 1 mM GRGDSP for 15 min as above, fixed for 60 min in 0.5% paraformaldehyde, then diluted and centrifuged to remove the peptide. The resuspended membranes were incubated with ^{125}I-fibrinogen (200 nM; ▨) or ^{125}I-mAb 10E5 (200 nM; ■). mAb 10E5 binding is a measure of the total amount of GP IIb–IIIa available for ligand binding.

detergent-solubilized GP IIb–IIIa to RGD peptides *irreversibly* converts the receptor into a fibrinogen-binding competent form. In contrast, in intact platelets, both RGD-induced [47,49] and agonist-induced [10] changes in the conformation of GP IIb–IIIa are *reversible*. The active form of the receptor on platelets can be preserved by paraformaldehyde fixation of the cells [14,50].

To determine whether the conformational changes associated with peptide occupancy of GP IIb–IIIa in membranes altered the receptor's ability to recognize fibrinogen, membranes were pretreated with 1 mM GRGDSP and assayed for fibrinogen binding. Membranes exposed to GRGDSP failed to bind substantial amounts of fibrinogen following removal of the peptide (Figure 10a). Binding and subsequent removal of the peptide was monitored by following ^{125}I-YRGDS binding (85% of bound peptide removed). Thus RGD peptides appear to interact with GP IIb–IIIa, but any conformational changes induced in the receptor are reversed upon removal of the peptide. However, membranes that were incubated with GRGDSP and subsequently fixed with paraformaldehyde displayed levels of fibrinogen binding equal to the total amount of GP IIb–IIIa in the membranes (Figure 10b). Fixation alone, in the absence of RGD peptide, did not promote fibrinogen binding (Figure 10b). When the conformational state of GP IIb–IIIa in the membranes was assessed with the activation-dependent mAb PAC-1, the results mirrored those obtained for fibrinogen binding to the membranes. Purified platelet membranes bound low levels of mAb PAC-1. The binding was inhibited by co-incubation with GRGDSP and enhanced by the 'activating' mAb 62 (Figure 11a, 'CTL'). The inhibitory effect of GRGDSP on PAC-1 binding was lost upon removal of the peptide (Figure 11a, 'Washed'). However, membranes that were pretreated with GRGDSP and fixed with paraformaldehyde bound high levels of PAC-1 following removal of the peptide (Figure 11a, 'Fixed'). Fixation alone slightly elevated PAC-1 binding. Thus synthetic peptides alter the conformation of GP IIb–IIIa in membranes such that the receptor assumes a reversible PAC-1- and fibrinogen-binding-competent form that can be maintained by membrane fixation.

The reversibility of RGD-induced changes in GP IIb–IIIa in intact platelets and isolated membranes but not in detergent-solubilized GP IIb–IIIa suggests that a lipid environment may influence the affinity state of GP IIb–IIIa. To investigate further the role of lipids on the conformation of GP IIb–IIIa, we examined the effect of GRGDSP on purified GP IIb–IIIa incorporated into phospholipid vesicles, where it has been previously reported that most of the GP IIb–IIIa does not bind fibrinogen [30]. When GP IIb–IIIa was incorporated into phospholipid vesicles, the effects of peptide on the conformation of receptor were also reversible and required paraformaldehyde fixation to be maintained (Figure 11b). As was the case for PAC-1 binding to membranes, basal PAC-1 binding to the vesicles was low and was enhanced by mAb 62 (Figure 11b, 'CTL'). Vesicles exposed to GRGDSP and washed to remove the peptide failed to bind high levels of PAC-1, unless the vesicles were fixed prior to the removal of the peptide (Figure 11b, 'Washed' and 'Fixed'). The exposure of LIBS 1-binding sites in purified GP IIb–IIIa incorporated into phospholipid vesicles was similarly reversed in

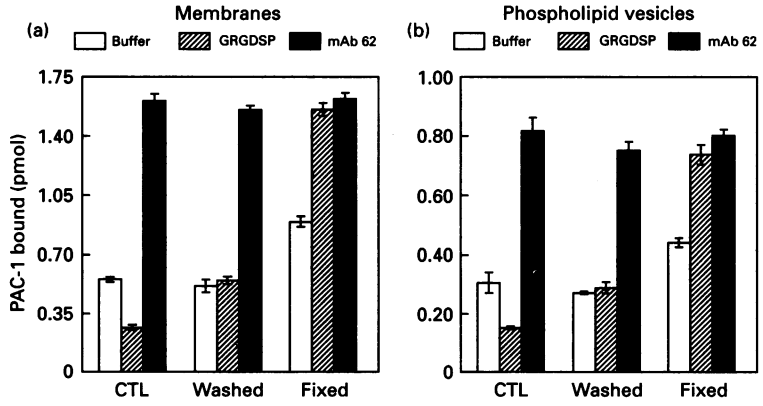

Figure 11 Induction of ^{125}I-mAb PAC-1 binding to purified platelet membranes and GP IIb–IIIa incorporated into phospholipid vesicles

Platelet membranes (**a**) and phospholipid vesicles containing GP IIb–IIIa (**b**) were incubated with buffer (□) or 1 mM GRGDSP (▨) for 15 min at room temperature. The effect of this treatment on the activation state of GP IIb–IIIa was assessed by measuring the binding of ^{125}I-mAb PAC-1 (80 nM) without peptide removal ('CTL'), after removal of the peptide ('Washed') or following fixation and peptide removal ('Fixed'). The GP IIb–IIIa 'activating' mAb 62 (5 μM; ■) was included in the binding reaction to maximally expose mAb PAC-1 binding sites under each of these conditions.

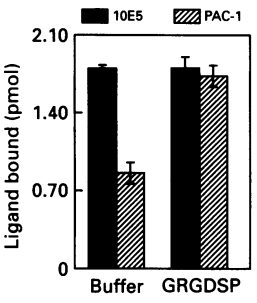

Figure 12 Peptide-induced changes in detergent-solubilized GP IIb–IIIa are retained after incorporation of the complex into phospholipid vesicles

GP IIb–IIIa in octyl glucoside buffer was incubated with buffer or 1 mM GRGDSP for 30 min at room temperature before the proteins were incorporated into phospholipid vesicles. The binding of ^{125}I-mAb 10E5 (■) or ^{125}I-mAb PAC-1 (▨) to phospholipid vesicles was measured. Results are shown as the amount of ^{125}I-ligand specifically bound. Non-specific binding was defined as the amount of ligand bound to phospholipid vesicles lacking GP IIb–IIIa.

the absence of peptide (results not shown). Thus our results indicate that, while we can induce a fibrinogen binding-competent form of GP IIb–IIIa in platelet membranes or phospholipid vesicles, the activated state of the receptor reverts to the resting state in the absence of a perturbing agent (e.g. membrane protein proteolysis, an activating mAb or paraformaldehyde fixation).

Since the exposure of fibrinogen-binding sites on GP IIb–IIIa in detergent solution by peptides has been reported to occur irreversibly [14,49], we sought to determine whether such changes in GP IIb–IIIa could be maintained following the incorporation of the protein into phospholipid vesicles. In these experiments, GP IIb–IIIa in octyl glucoside-containing buffer was incubated with GRGDSP *before* incorporation into phospholipid vesicles. Under these conditions, an increase in mAb PAC-1 binding was observed to phospholipid vesicles containing GP IIb–IIIa that had been pre-exposed to peptide (Figure 12). These results suggest that the effects of GRGDSP on the conformation of GP IIb–IIIa depends on the environment surrounding the receptor at the time of peptide treatment.

Regulation of the affinity state of GP IIb–IIIa in intact platelets may require the concerted actions of elements that promote fibrinogen binding to the receptor, as well as factors that return the receptor to the resting state. In platelets, agonist-exposed fibrinogen-binding sites lose the capacity to bind over time, and the closure of sites can be enhanced by elevating intra-platelet cyclic AMP levels [10]. Our results suggest that a lipid environment stabilizes the resting state of the receptor. Thus the disappearance of the fibrinogen-binding-competent form of GP IIb–IIIa in agonist-treated platelets and RGD-treated membranes/phospholipid vesicles may be the result of an intrinsic ability of the receptor in a lipid environment to revert to the resting state. In contrast, when activated with peptides, detergent-solubilized GP IIb–IIIa does not spontaneously revert to a resting state when inserted into phospholipid vesicles (Figure 12). One explanation for these findings is that peptide binding to detergent-solubilized GP IIb–IIIa induces different or additional conformational changes in the receptor that do not occur when peptides bind to GP IIb–IIIa in platelet membranes or in phospholipid vesicles. Alternatively, we cannot exclude the possibility that, in intact platelets as well as in platelet membranes and phospholipid vesicles containing purified GP IIb–IIIa, additional unidentified regulatory factors act upon GP IIb–IIIa to return the receptor to the resting state. In this scenario, the peptide-activated form of detergent solubilized GP IIb–IIIa could be maintained following incorporation into phospholipid vesicles if peptide binding to solubilized GP IIb–IIIa released a negative regulatory factor. Loss of a factor during vesicle formation could account for the retention of the activated state of GP IIb–IIIa. In this regard, O'Toole et al. [17] demonstrated that the cytoplasmic domain of GP IIb is required to maintain the receptor in the resting state in intact cells and suggested that this region of GP IIb interacts with an intracellular regulatory moiety which controls the affinity state of the receptor.

In summary, we have found that ^{125}I-fibrinogen binds with high affinity to a single class of sites in isolated platelet membranes, presumably GP IIb–IIIa. However, the fibrinogen-binding capacity represents only a fraction of the total GP IIb–IIIa in the membranes. Fibrinogen binding can be enhanced by pretreatment of the membranes with either the serine proteinase chymotrypsin or a GP IIb–IIIa activating mAb. Platelet activators, including agonists, G-protein activators and PKC, do not stimulate fibrinogen binding, suggesting that one or more cytosolic regulatory components may be lost during the membrane isolation procedure. Platelet membranes possess the ability to bind substantial amounts of YRGDS, and the maximal binding capacity for YRGDS is similar to the total amount of GP IIb–IIIa. Binding of RGD peptides to GP IIb–IIIa appears to convert the receptor into a ligand-binding-competent state that can be retained by membrane fixation but which is reversed in the absence of fixation. Thus the low levels of fibrinogen binding to the membranes could be explained by an absence of a GP IIb–IIIa activating factor and/or the presence of a GP IIb–IIIa repressing activity. Our data suggest that a lipid environment may be one mechanism that stabilizes the resting state of the receptor. Further investigations in this system may provide direct evidence for the existence of factors which modulate the affinity of GP IIb–IIIa for fibrinogen.

Activation of the fibrinogen-binding properties of GP IIb–IIIa represents a crucial step in the process of platelet aggregation. Understanding the molecular mechanisms that regulate this event requires the development of experimental approaches for evaluating the activity of GP IIb–IIIa. Shattil and Brass [51,52] have characterized the exposure of binding sites on GP IIb–IIIa in platelets permeabilized with saponin or complement proteins for this purpose. The data presented here suggest that isolated platelet membranes may provide another system in which regulation of GP IIb–IIIa can be investigated. The ability to examine the fibrinogen-binding capacity of purified platelet membranes should allow for further dissection of the platelet signalling events that are required for modulation of the affinity state of GP IIb–IIIa.

We thank Dr. Mark Ginsberg for his advice and encouragement, Dr. Andrew Morris and Anne Criss for their many helpful discussions and their technical expertise, and Michael Springle for his help with platelet preparations. We are indebted to Dr. Peter Newman for mAbs AP3 and AP4; to Dr. Barry Coller for mAb 10E5; to Dr. Mark Ginsberg for mAbs LIBS1, PMI-1 and 62; to Dr. David Phillips for polyclonal antibodies against GP IIb–IIIa; and to Dr. Sanford Shattil for mAb PAC-1. This work was supported by National Institute of Health grant R29HL38405. Additional support was provided by a Grant-in-Aid from the American Heart Association (L.V.P.), a fellowship from the Pharmaceutical Manufacturer's Association (S.S.S.) and predoctoral training grant GM07040 (S.S.S.). L.V.P. is an Established Investigator of the American Heart Association.

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Received 30 October 1992/6 January 1993; accepted 13 January 1993